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(54) Title: ALKALINE LIPOLYTIC ENZYME

(57) Abstract

An alkaline lipolytic enzyme can be derived from fungal strains of *Botryosphaeria or Guignardia*, two closely related genera not previously reported to produce lipolytic enzyme. The novel lipolytic enzyme has optimum activity around pH 10, making it well suited for use in detergents. Advantageously, the microorganisms are *Ascomycetes* for which suitable expression systems are well developed.

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ALKALINE LIPOLYTIC ENZYME

TECHNICAL FIELD

This invention relates to an alkaline lipolytic enzyme, to a lipolytic enzyme-producing microbial strain, to methods for the production of lipolytic enzyme and to a detergent composition comprising the lipolytic enzyme.

BACKGROUND ART

For a number of years lipolytic enzymes have been used as detergent additives to remove lipid or fatty stains from clothes and other textiles.

Thus, the prior art suggests the use of various microbial lipases as detergent additives. Examples include lipases derived from *Humicola lanuginosa* (also called *Thermomyces lanuginosus*, EP 258 068 and EP 305 216), *Rhizomucor miehei* (EP 238 023), *Candida antarctica* (EP 214 761), various species of *Pseudomonas* such as *P. alcaligenes* and *P. pseudoalcaligenes* (EP 218 272), *P. cepacia* (EP 331 376), *Bacillus*, e.g. *B. subtilis* (Dartois et al., (1993) Biochemica et Biophysica Acta 1131, 253-260), *B. stearothermophilus* (JP-A 64-74992) and *B. pumilus* (WO 91/16422).

For commercial production of enzymes such as lipases, it is preferred to express the enzyme in a suitable host organism for higher yield. Various expression systems are available, including expression of enzymes from *Ascomycetes* in *Aspergillus* (EP 238 023).

Many detergents are alkaline with a high pH in solution (e.g. around pH 10), so there is a need for lipolytic enzymes with high activity at high pH. The lipolytic enzyme should be derived from a type of microorganism for which suitable expression systems are available.

SUMMARY OF THE INVENTION

Surprisingly, we have found that an alkaline lipolytic enzyme can be derived from fungal strains of *Botryosphaeria or Guignardia*, two closely related genera not previously reported to produce lipolytic enzyme. The novel lipolytic enzyme has optimum activity around pH 10, making it well suited for use in

detergents. Advantageously, the microorganisms are *Ascomycetes* for which suitable expression systems are well developed.

Accordingly, the invention provides a lipolytic enzyme which is derived from a strain of *Botryosphaeria or Guignardia* or is immunologically reactive with an antibody raised against a purified lipolytic enzyme produced by such strain, and has optimum activity at a pH in the range 9-11 in the presence of 50 mM Ca^{++}.

Another aspect of the invention provides an alkaline lipolytic enzyme which contains an amino acid sequence selected from the group consisting of SEQ ID NO: 1, 2, 3, 4 and 5.

The invention also provides novel lipolytic enzyme-producing strains of *Botryosphaeria* as a biologically pure culture.

In another aspect, the invention provides a process for producing an alkaline lipolytic enzyme, comprising cultivation of a lipolytic enzyme-producing strain of *Botryosphaeria* or *Guignardia* in a suitable nutrient medium, followed by recovery of the alkaline lipolytic enzyme.

The invention further provides a method for producing an alkaline lipolytic enzyme, comprising:

a) isolating a DNA fragment encoding the lipolytic enzyme from a lipolytic enzyme-producing strain of *Botryosphaeria or Guignardia*,

b) combining the DNA fragment with appropriate expression signal(s) in an appropriate vector,

c) introducing the vector or parts thereof into an appropriate host,

d) cultivating the host organism under conditions leading to expression of the lipolytic enzyme, and

e) recovering the lipolytic enzyme from the culture medium.

Finally, the invention provides a detergent composition comprising a surfactant together with an effective amount of said lipolytic enzyme.

BRIEF DESCRIPTION OF THE DRAWINGS

Figures 1 and 2 show graphs of lipolytic enzyme activity vs. pH for lipolytic enzymes produced by *Botryosphaeria sp.* CBS 102.95 and *B. ribis* CBS 504.94, respectively.

DETAILED DISCLOSURE OF THE INVENTION

Microorganisms

The microbial strains used in this invention belong to the genera *Botryosphaeria* or *Guignardia*. The two genera are closely related and were considered synonyms by M.E. Barr (Contrib. Univ. Mich. Herb., 9: 523-638, 1972), but most authors consider them separate genera - see for instance A. Sivanesan, J. Cramer, Vaduz, 701 pp, 1984.

Both genera are described by Richard T. Hanlin, Illustrated Genera of Ascomycetes, APS Press, The American Phytopathological Society, St. Paul, Minnesota, 1990, p. 46-49. The genus *Botryosphaeria* is also described by Punithalingam, E. & Holliday, P. (1973) *CMI Descriptions of Pathogenic Fungi and Bacteria* No. 395. When cultivated, the strains may develop in the *Fusicoccum* state or the microconidial state.

Two strains have been isolated and deposited by the inventors according to the Budapest Treaty on the International Recognition of the Deposit of Microorganisms for the Purposes of Patent Procedure at Centraalbureau Voor Schimmelcultures (CBS), Oosterstraat 1, Postbus 273, NL-3740 AG Baarn, Netherlands. The two strains were classified by standard taxonomic methods.

The deposit data and the taxonomic identification of the strains were as follows:

Depositors' reference	NN143554	NN115210
Deposit number	CBS 102.95	CBS 504.94
Deposit date	January 6, 1995	October 19, 1994
Taxonomic designation	*Botryosphaeria sp.*	*Botryosphaeria ribis*

Also, the following publicly available strains can be used in the invention:

Genus	Species	Inventors' strain No.	Deposit number
Botryosphaeria	*B. berengeriana*	NN102565	MAFF 06-45001
	B. berengeriana f. sp. pilicola	NN102566	MAFF 06-45002
	B. dothidea	NN102558	JCM 2733
		NN102559	JCM 2735
		NN102560	JCM 2736
		NN102561	JCM 2737
		NN102562	JCM 2738
	B. parva	NN103321	ATCC 58191
	B. ribis	NN103322	ATCC 56125
	B. ribis var. chromogena	NN103313	CBS 121.26
	B. xanthocephala	NN103324	ATCC 60638
Guignardia	*G. laricina*	NN102563	IFO 7887
		NN102564	IFO 7888
	G. paulowniae	NN102567	MAFF 03-05151

The above-mentioned strains are freely available from the following depositary institutions for microorganisms:

MAFF: Ministry of Agriculture, Forestry and Fisheries, National Institute of Agro-Biological Research, 1-2 Kannon-dai 2-chome, Tsukuba, Ibaraki 305, Japan.

JCM: Japan Collection of Microorganisms, RIKEN, Wako, Saitama 351-01, Japan.

IFO: Institute for Fermentation, Osaka, 17-85, Juso-honmachi 2-chome, Yodogawa-ku, Osaka 532, Japan.

CBS: Centraal Bureau voor Schimmelcultures, Oosterstraat 1, 3740 AG Baarn, Netherlands.

ATCC: American Type Culture Collection, 12301 Parklawn Drive, Rockville, Maryland 20852, USA.

Variants and mutants of these strains, capable of producing lipolytic enzyme, may also be used in the invention.

Production of lipolytic enzyme

Lipolytic enzyme may be produced by cultivating any of the microorganisms described above in a suitable nutrient medium, optionally followed by recovery and purification, according to methods well known in the art or as described in the examples of this specification.

The lipolytic enzyme may also be obtained by recombinant DNA-technology by methods known in the art *per se*, e.g. isolating a DNA fragment encoding the lipolytic enzyme, combining the DNA fragment with appropriate expression signal(s) in an appropriate vector, introducing the vector or parts thereof into an appropriate host (e.g. a filamentous fungus, preferably a member of the genus *Aspergillus*), either as an autonomously replicating plasmid or integrated into the chromosome, cultivating the host organism under conditions leading to expression of the lipolytic enzyme, and recovering the lipolytic enzyme from the culture medium.

The isolation of a DNA sequence may be done by so-called expression cloning, comprising the following steps:

a) cloning, in suitable vectors, a cDNA library from a lipolytic enzyme-producing strain of *Botryosphaeria or Guignardia*,

b) transforming suitable yeast host cells with said vectors,

c) cultivating the transformed yeast host cells under suitable conditions to express the alkaline lipolytic enzyme,

d) screening for positive clones by determining the lipolytic enzyme activity expressed in step (c).

The expression cloning may be done as described in WO 93/11249 or in H. Dalbøge and H.P. Heldt-Hansen, *Mol. Gen. Genet.* (1994) 243:253-260. A preferred heterologous host cell is a strain of *Aspergillus*, e.g. *A. oryzae* or *A. niger*, e.g. using the expression system described in EP-A-0 238 023.

After the cultivation, the lipolytic enzyme may be recovered and purified from the culture broth by conventional methods, such as hydrophobic chromatography, ion exchange chromatography and combinations thereof.

Lipolytic enzymes

The enzymes of this invention are lipolytic enzymes. In the present context the term "lipolytic enzyme" is intended to indicate an enzyme classified under the Enzyme Classification number E.C. 3.1.1.- (Carboxylic Ester Hydrolases) in accordance with the Recommendations (1992) of the International Union of Biochemistry and Molecular Biology (IUBMB). Lipolytic enzymes thus exhibit hydrolytic activity towards at least one of the types of ester bonds mentioned in the context of E.C. 3.1.1. The lipolytic enzymes of the invention preferably have lipase activity (with triglycerides as substrate).

Enzymatic properties

The lipolytic enzymes of this invention are characterized by high activity at alkaline pH. More specifically, they have optimum activity at a pH higher than 9, particularly in the range 9-11, in the presence of 50 mM Ca^{++}.

Figures 1 and 2 show graphs of lipolytic enzyme activity vs. pH for lipolytic enzymes produced by *Botryosphaeria sp.* CBS 102.95 and *B. ribis* CBS 504.94, respectively. Both lipolytic enzymes have optimum activity at about pH 10.

The isoelectric point is 3.8 for lipolytic enzyme from CBS 102.95 and 3.4 for lipolytic enzyme from CBS 504.94. The molecular weight is 55,000 Dalton for lipolytic enzyme from CBS 102.95 and 64,000 for lipolytic enzyme from CBS 504.94. The specific lipolytic enzyme activity is 400 LU/A_{280} for lipolytic enzyme from CBS 102.95 and 300 LU/A_{280} for lipolytic enzyme from CBS 504.94 (A_{280} = mg of protein determined from absorbance at 280 nm).

One Lipolytic enzyme Unit (LU) is the amount of enzyme which, under standard conditions (i.e. at 30.0°C; pH 7.0; and tributyrin substrate) liberates 1 μmol of titratable butyric acid per minute.

Immunochemical Properties

Alkaline lipolytic enzymes having immunochemical properties identical or partially identical to those of a lipolytic enzyme native to a strain of *Botryosphaeria or Guignardia* and having the stated properties are within the scope of the invention.

The immunochemical properties can be determined by immunological cross-reaction identity tests. The identity tests can be performed by the well-known Ouchterlony double immunodiffusion procedure or by tandem crossed immunoelectrophoresis according to I. M. Roitt; Immunology, Gower Medical Publishing (1985) and N. H. Axelsen; Handbook of Immunoprecipitation-in-Gel Techniques, Blackwell Scientific Publications (1983), Chapters 5 and 14. The terms immunochemical identity (antigenic identity) and partial immunochemical identity (partial antigenic identity) are described in Axelsen, *supra*, Chapters 5, 19 and 20 and Roitt, *supra*, Chapter 6.

Monospecific antiserum for use in immunological tests can be raised, e.g. in rabbits, against a purified lipolytic enzyme, e.g. as described in Chapter 41 of N.H. Axelsen, *supra* or Chapter 23 of N.H. Axelsen et al., A Manual of Quantitative Immunoelectrophoresis, Blackwell Scientific Publications (1973).

Detergent additive

According to the invention, the lipolytic enzyme may typically be used as an additive in a detergent composition. This additive is conveniently formulated as a non-dusting granulate, a stabilized liquid, a slurry or a protected enzyme.

A suitable activity range for a detergent additive containing the lipolytic enzyme of this invention is 0.01-100 mg of pure enzyme protein per g of the detergent additive

Detergent

The lipolytic enzyme of the invention may be incorporated in concentrations conventionally employed in detergents. The amount of lipolytic enzyme protein may be 0.001-10 mg per gram of detergent or 0.001-100 mg per liter of wash liquor.

Detergent Compositions

According to the invention, the lipolytic enzyme may typically be a component of a detergent composition. As such, it may be included in the detergent composition in the form of a non-dusting granulate, a stabilized liquid, or a protected enzyme. Non-dusting granulates may be produced, e.g., as disclosed in US 4,106,991 and 4,661,452 (both to Novo Industri A/S) and may optionally be coated by methods known in the art. Examples of waxy coating materials are poly(ethylene oxide) products (polyethylene glycol, PEG) with mean molecular weights of 1000 to 20000; ethoxylated nonylphenols having from 16 to 50 ethylene oxide units; ethoxylated fatty alcohols in which the alcohol contains from 12 to 20 carbon atoms and in which there are 15 to 80 ethylene oxide units; fatty alcohols; fatty acids; and mono- and di- and triglycerides of fatty acids. Examples of film-forming coating materials suitable for application by fluid bed techniques are given in patent GB 1483591. Liquid enzyme preparations may, for instance, be stabilized by adding a polyol such as propylene glycol, a sugar or sugar alcohol, lactic acid or boric acid according to established methods. Other enzyme stabilizers are well known in the art. Protected enzymes may be prepared according to the method disclosed in EP 238,216.

The detergent composition of the invention may be in any convenient form, e.g. as powder, granules, paste or liquid. A liquid detergent may be aqueous, typically containing up to 70% water and 0-30% organic solvent, or nonaqueous.

The detergent composition comprises one or more surfactants, each of which may be anionic, nonionic, cationic, or zwitterionic. The detergent will usually contain 0-50% of anionic surfactant such as linear alkylbenzene sulfonate (LAS), alpha-olefin sulfonate (AOS), alkyl sulfate (fatty alcohol sulfate) (AS), alcohol ethoxysulfate (AEOS or AES), secondary alkane sulfonates (SAS), alpha-sulfo fatty

acid methyl esters, alkyl- or alkenylsuccinic acid, or soap. It may also contain 0-40% of nonionic surfactant such as alcohol ethoxylate (AEO or AE), carboxylated alcohol ethoxylates, nonylphenol ethoxylate, alkylpolyglycoside, alkyldimethylamine oxide, ethoxylated fatty acid monoethanolamide, fatty acid monoethanolamide, or polyhydroxy alkyl fatty acid amide (e.g. as described in WO 92/06154).

The detergent composition may additionally comprise one or more other enzymes, such as amylase, cutinase, protease, cellulase, peroxidase, and oxidase, e.g., laccase.

The detergent may contain 1-65% of a detergent builder or complexing agent such as zeolite, diphosphate, triphosphate, phosphonate, citrate, nitrilotriacetic acid (NTA), ethylenediaminetetraacetic acid (EDTA), diethylenetriaminepentaacetic acid (DTMPA), alkyl- or alkenylsuccinic acid, soluble silicates or layered silicates (e.g. SKS-6 from Hoechst). The detergent may also be unbuilt, i.e. essentially free of detergent builder.

The detergent may comprise one or more polymers. Examples are carboxymethyl cellulose (CMC), poly(vinyl pyrrolidone) (PVP), polyethylene glycol (PEG), poly(vinyl alcohol) (PVA), polycarboxylates such as polyacrylates, maleic/acrylic acid copolymers and lauryl methacrylate/acrylic acid copolymers.

The detergent may contain a bleaching system which may comprise a H_2O_2 source such as perborate or percarbonate which may be combined with a peracid-forming bleach activator such as tetraacetylethylenediamine (TAED) or nonanoyloxybenzene sulfonate (NOBS). Alternatively, the bleaching system may comprise peroxy acids of, e.g., the amide, imide, or sulfone type.

The enzymes of the detergent composition of the invention may be stabilized using conventional stabilizing agents, e.g. a polyol such as propylene glycol or glycerol, a sugar or sugar alcohol, lactic acid, boric acid, or a boric acid derivative such as, e.g., an aromatic borate ester, and the composition may be formulated as described in, e.g., WO 92/19709 and WO 92/19708.

The detergent may also contain other conventional detergent ingredients such as, e.g., fabric conditioners including clays, foam boosters, suds suppressors, anti-corrosion agents, soil-suspending agents, anti-soil-redeposition agents, dyes, bactericides, optical brighteners, or perfume.

The pH (measured in aqueous solution at use concentration) will usually be neutral or alkaline, e.g. in the range of 7-11.

Particular forms of detergent compositions within the scope of the invention include:

1) A detergent composition formulated as a granulate having a bulk density of at least 600 g/l comprising

Linear alkylbenzene sulfonate (calculated as acid)	7 - 12%
Alcohol ethoxysulfate (e.g. C_{12-18} alcohol, 1-2 EO) or alkyl sulfate (e.g. C_{16-18})	1 - 4%
Alcohol ethoxylate (e.g. C_{14-15} alcohol, 7 EO)	5 - 9%
Sodium carbonate (as Na_2CO_3)	14 - 20%
Soluble silicate (as $Na_2O,2SiO_2$)	2 - 6%
Zeolite (as $NaAlSiO_4$)	15 - 22%
Sodium sulfate (as Na_2SO_4)	0 - 6%
Sodium citrate/citric acid (as $C_6H_5Na_3O_7/C_6H_8O_7$)	0 - 15%
Sodium perborate (as $NaBO_3.H_2O$)	11 - 18%
TAED	2 - 6%
Carboxymethyl cellulose	0 - 2%
Polymers (e.g. maleic/acrylic acid copolymer, PVP, PEG)	0 - 3%
Enzymes (calculated as pure enzyme protein)	0.0001 - 0.1%
Minor ingredients (e.g. suds suppressors, perfume, optical brightener, photobleach)	0 - 5%

2) A detergent composition formulated as a granulate having a bulk density of at least 600 g/l comprising

Linear alkylbenzene sulfonate (calculated as acid)	6 - 11%
Alcohol ethoxysulfate (e.g. C_{12-18} alcohol, 1-2 EO or alkyl sulfate (e.g. C_{16-18})	1 - 3%
Alcohol ethoxylate (e.g. C_{14-15} alcohol, 7 EO)	5 - 9%
Sodium carbonate (as Na_2CO_3)	15 - 21%

Soluble silicate (as $Na_2O,2SiO_2$)	1 - 4%
Zeolite (as $NaAlSiO_4$)	24 - 34%
Sodium sulfate (as Na_2SO_4)	4 - 10%
Sodium citrate/citric acid (as $C_6H_5Na_3O_7/C_6H_8O_7$)	0 - 15%
Carboxymethyl cellulose	0 - 2%
Polymers (e.g. maleic/acrylic acid copolymer, PVP, PEG)	1 - 6%
Enzymes (calculated as pure enzyme protein)	0.0001 - 0.1%
Minor ingredients (e.g. suds suppressors, perfume)	0 - 5%

3) A detergent composition formulated as a granulate having a bulk density of at least 600 g/l comprising

Linear alkylbenzene sulfonate (calculated as acid)	5 - 9%
Alcohol ethoxylate (e.g. C_{12-15} alcohol, 7 EO)	7 - 14%
Soap as fatty acid (e.g. C_{16-22} fatty acid)	1 - 3%
Sodium carbonate (as Na_2CO_3)	10 - 17%
Soluble silicate (as $Na_2O,2SiO_2$)	3 - 9%
Zeolite (as $NaAlSiO_4$)	23 - 33%
Sodium sulfate (as Na_2SO4)	0 - 4%
Sodium perborate (as $NaBO_3.H_2O$)	8 - 16%
TAED	2 - 8%
Phosphonate (e.g. EDTMPA)	0 - 1%
Carboxymethyl cellulose	0 - 2%
Polymers (e.g. maleic/acrylic acid copolymer, PVP, PEG)	0 - 3%
Enzymes (calculated as pure enzyme protein)	0.0001 - 0.1%
Minor ingredients (e.g. suds suppressors, perfume, optical brightener)	0 - 5%

4) A detergent composition formulated as a granulate having a bulk density of at least 600 g/l comprising

Linear alkylbenzene sulfonate (calculated as acid)	8 - 12%
Alcohol ethoxylate (e.g. C_{12-15} alcohol, 7 EO)	10 - 25%
Sodium carbonate (as Na_2CO_3)	14 - 22%
Soluble silicate (as $Na_2O.2SiO_2$)	1 - 5%
Zeolite (as $NaAlSiO_4$)	25 - 35%
Sodium sulfate (as Na_2SO_4)	0 - 10%
Carboxymethyl cellulose	0 - 2%
Polymers (e.g. maleic/acrylic acid copolymer, PVP, PEG)	1 - 3%
Enzymes (calculated as pure enzyme protein)	0.0001 - 0.1%
Minor ingredients (e.g. suds suppressors, perfume)	0 - 5%

5) An aqueous liquid detergent composition comprising

Linear alkylbenzene sulfonate (calculated as acid)	15 - 21%
Alcohol ethoxylate (e.g. C_{12-15} alcohol, 7 EO or C_{12-15} alcohol, 5 EO)	12 - 18%
Soap as fatty acid (e.g. oleic acid)	3 - 13%
Alkenylsuccinic acid (C_{12-14})	0 - 13%
Aminoethanol	8 - 18%
Citric acid	2 - 8%
Phosphonate	0 - 3%
Polymers (e.g. PVP, PEG)	0 - 3%
Borate (as B_4O_7)	0 - 2%
Ethanol	0 - 3%
Propylene glycol	8 - 14%
Enzymes (calculated as pure enzyme protein)	0.0001 - 0.1%
Minor ingredients (e.g. dispersants, suds suppressors, perfume, optical brightener)	0 - 5%

6) An aqueous structured liquid detergent composition comprising

Linear alkylbenzene sulfonate (calculated as acid)	15 - 21%
Alcohol ethoxylate (e.g. C_{12-15} alcohol, 7 EO, or C_{12-15} alcohol, 5 EO)	3 - 9%
Soap as fatty acid (e.g. oleic acid)	3 - 10%
Zeolite (as $NaAlSiO_4$)	14 - 22%
Potassium citrate	9 - 18%
Borate (as B_4O_7)	0 - 2%
Carboxymethyl cellulose	0 - 2%
Polymers (e.g. PEG, PVP)	0 - 3%
Anchoring polymers such as, e.g., lauryl methacrylate/acrylic acid copolymer; molar ratio 25:1; MW 3800	0 - 3%
Glycerol	0 - 5%
Enzymes (calculated as pure enzyme protein)	0.0001 - 0.1%
Minor ingredients (e.g. dispersants, suds suppressors, perfume, optical brighteners)	0 - 5%

7) A detergent composition formulated as a granulate having a bulk density of at least 600 g/l comprising

Fatty alcohol sulfate	5 - 10%
Ethoxylated fatty acid monoethanolamide	3 - 9%
Soap as fatty acid	0 - 3%
Sodium carbonate (as Na_2CO_3)	5 - 10%
Soluble silicate (as $Na_2O.2SiO_2$)	1 - 4%
Zeolite (as $NaAlSiO_4$)	20 - 40%
Sodium sulfate (as Na_2SO_4)	2 - 8%
Sodium perborate (as $NaBO_3.H_2O$)	12 - 18%
TAED	2 - 7%
Polymers (e.g. maleic/acrylic acid copolymer, PEG)	1 - 5%

Enzymes (calculated as pure enzyme protein)	0.0001 - 0.1%
Minor ingredients (e.g. optical brightener, suds suppressors, perfume)	0 - 5%

8) A detergent composition formulated as a granulate comprising

Linear alkylbenzene sulfonate (calculated as acid)	8 - 14%
Ethoxylated fatty acid monoethanolamide	5 - 11%
Soap as fatty acid	0 - 3%
Sodium carbonate (as Na_2CO_3)	4 - 10%
Soluble silicate (as $Na_2O.2SiO_2$)	1 - 4%
Zeolite (as $NaAlSiO_4$)	30 - 50%
Sodium sulfate (as Na_2SO_4)	3 - 11%
Sodium citrate (as $C_6H_5Na_3O_7$)	5 - 12%
Polymers (e.g. PVP, maleic/acrylic acid copolymer, PEG)	1 - 5%
Enzymes (calculated as pure enzyme protein)	0.0001 - 0.1%
Minor ingredients (e.g. suds suppressors, perfume)	0 - 5%

9) A detergent composition formulated as a granulate comprising

Linear alkylbenzene sulfonate (calculated as acid)	6 - 12%
Nonionic surfactant	1 - 4%
Soap as fatty acid	2 - 6%
Sodium carbonate (as Na_2CO_3)	14 - 22%
Zeolite (as $NaAlSiO_4$)	18 - 32%
Sodium sulfate (as Na_2SO_4)	5 - 20%
Sodium citrate (as $C_6H_5Na_3O_7$)	3 - 8%
Sodium perborate (as $NaBO_3.H_2O$)	4 - 9%
Bleach activator (e.g. NOBS or TAED)	1 - 5%
Carboxymethyl cellulose	0 - 2%
Polymers (e.g. polycarboxylate or PEG)	1 - 5%

Linear alkylbenzene sulfonate (calculated as acid)	6 - 12%
Enzymes (calculated as pure enzyme protein)	0.0001 - 0.1%
Minor ingredients (e.g. optical brightener, perfume)	0 - 5%

10) An aqueous liquid detergent composition comprising

Linear alkylbenzene sulfonate (calculated as acid)	15 - 23%
Alcohol ethoxysulfate (e.g. C_{12-15} alcohol, 2-3 EO)	8 - 15%
Alcohol ethoxylate (e.g. C_{12-15} alcohol, 7 EO, or C_{12-15} alcohol, 5 EO)	3 - 9%
Soap as fatty acid (e.g. lauric acid)	0 - 3%
Aminoethanol	1 - 5%
Sodium citrate	5 - 10%
Hydrotrope (e.g. sodium toluene sulfonate)	2 - 6%
Borate (as B_4O_7)	0 - 2%
Carboxymethyl cellulose	0 - 1%
Ethanol	1 - 3%
Propylene glycol	2 - 5%
Enzymes (calculated as pure enzyme protein)	0.0001 - 0.1%
Minor ingredients (e.g. polymers, dispersants, perfume, optical brighteners)	0 - 5%

11) An aqueous liquid detergent composition comprising

Linear alkylbenzene sulfonate (calculated as acid)	20 - 32%
Alcohol ethoxylate (e.g. C_{12-15} alcohol, 7 EO, or C_{12-15} alcohol, 5 EO)	6 - 12%
Aminoethanol	2 - 6%
Citric acid	8 - 14%
Borate (as B_4O_7)	1 - 3%
Polymer (e.g. maleic/acrylic acid copolymer, anchoring polymer such as, e.g., lauryl methacrylate/acrylic acid copolymer)	0 - 3%

Linear alkylbenzene sulfonate (calculated as acid)	20 - 32%
Glycerol	3 - 8%
Enzymes (calculated as pure enzyme protein)	0.0001 - 0.1%
Minor ingredients (e.g. hydrotropes, dispersants, perfume, optical brighteners)	0 - 5%

12) A detergent composition formulated as a granulate having a bulk density of at least 600 g/l comprising

Anionic surfactant (linear alkylbenzene sulfonate, alkyl sulfate, alpha-olefin sulfonate, alpha-sulfo fatty acid methyl esters, alkane sulfonates, soap)	25 - 40%
Nonionic surfactant (e.g. alcohol ethoxylate)	1 - 10%
Sodium carbonate (as Na_2CO_3)	8 - 25%
Soluble silicates (as Na_2O, $2SiO_2$)	5 - 15%
Sodium sulfate (as Na_2SO_4)	0 - 5%
Zeolite (as $NaAlSiO_4$)	15 - 28%
Sodium perborate (as $NaBO_3.4H_2O$)	0 - 20%
Bleach activator (TAED or NOBS)	0 - 5%
Enzymes (calculated as pure enzyme protein)	0.0001 - 0.1%
Minor ingredients (e.g. perfume, optical brighteners)	0 - 3%

13) Detergent formulations as described in 1) - 12) wherein all or part of the linear alkylbenzene sulfonate is replaced by (C_{12}-C_{18}) alkyl sulfate.

14) A detergent composition formulated as a granulate having a bulk density of at least 600 g/l comprising

(C_{12}-C_{18}) alkyl sulfate	9 - 15%
Alcohol ethoxylate	3 - 6%
Polyhydroxy alkyl fatty acid amide	1 - 5%
Zeolite (as $NaAlSiO_4$)	10 - 20%

(C_{12}-C_{18}) alkyl sulfate	9 - 15%
Layered disilicate (e.g. SK56 from Hoechst)	10 - 20%
Sodium carbonate (as Na_2CO_3)	3 - 12%
Soluble silicate (as $Na_2O.2SiO_2$)	0 - 6%
Sodium citrate	4 - 8%
Sodium percarbonate	13 - 22%
TAED	3 - 8%
Polymers (e.g. polycarboxylates and PVP=	0 - 5%
Enzymes (calculated as pure enzyme protein)	0.0001 - 0.1%
Minor ingredients (e.g. optical brightener, photo bleach, perfume, suds suppressors)	0 - 5%

15) A detergent composition formulated as a granulate having a bulk density of at least 600 g/l comprising

(C_{12}-C_{18}) alkyl sulfate	4 - 8%
Alcohol ethoxylate	11 - 15%
Soap	1 - 4%
Zeolite MAP or zeolite A	35 - 45%
Sodium carbonate (as Na_2CO_3)	2 - 8%
Soluble silicate (as $Na_2O.2SiO_2$)	0 - 4%
Sodium percarbonate	13 - 22%
TAED	1 - 8%
Carboxymethyl cellulose	0 - 3%
Polymers (e.g. polycarboxylates and PVP)	0 - 3%
Enzymes (calculated as pure enzyme protein)	0.0001 - 0.1%
Minor ingredients (e.g. optical brightener, phosphonate, perfume)	0 - 3%

16) Detergent formulations as described in 1) - 15) which contain a stabilized or encapsulated peracid, either as an additional component or as a substitute for already specified bleach systems.

17) Detergent compositions as described in 1), 3), 7), 9) and 12) wherein perborate is replaced by percarbonate.

18) Detergent compositions as described in 1), 3), 7), 9), 12), 14) and 15) which additionally contain a manganese catalyst. The manganese catalyst may, e.g., be one of the compounds described in "Efficient manganese catalysts for low-temperature bleaching", Nature 369, 1994, pp. 637-639.

19) Detergent composition formulated as a nonaqueous detergent liquid comprising a liquid nonionic surfactant such as, e.g., linear alkoxylated primary alcohol, a builder system (e.g. phosphate), enzyme and alkali. The detergent may also comprise anionic surfactant and/or a bleach system.

The lipolytic enzyme of the invention may be incorporated in concentrations conventionally employed in detergents. It is at present contemplated that, in the detergent composition of the invention, the lipolytic enzyme may be added in an amount corresponding to 0.00001-1 mg (calculated as pure enzyme protein) of lipolytic enzyme per liter of wash liquor.

EXAMPLES

Example 1

Production of lipolytic enzyme from *Botryosphaeria sp.*

The following media were used in this example:

Medium	YS-2	YS-25
pH	6.5	6.5
Glucose	20 g/L	5 g/L
Yeast extract	10 g/L	10 g/L
Peptone	10 g/L	10 g/L
K_2HPO_4	5 g/L	5 g/L
$MgSO_4 \cdot 7H_2O$	1 g/L	1 g/L
Soybean oil	0	2 ml/100 ml

A seed culture was prepared by inoculating strain CBS 102.95 from a slant of PDA (product of Difco) to two shake flasks containing 100 ml of YS-2 medium, and incubating the shake flasks with shaking for 2 days at 27°C. The final pH was 7.5.

A main culture was prepared by using the seed culture to inoculate 50 shake flasks with 100 ml of YS-25 medium and incubating for 2 days at 27°C with shaking. The final pH was 7.3.

2,900 ml of cell-free broth with a lipolytic enzyme activity of 17 LU/ml was recovered after removal of the cell mass. This was deionized and freeze-dried to obtain 11.9 g of powder sample with a lipolytic enzyme activity of 3,540 LU/g.

Example 2

Production of lipolytic enzyme from *Botryosphaeria ribis*

Lipolytic enzyme was produced in the same manner as in Example 1, except that strain CBS 504.94 was used, and 3 days were used for the main culture. A cell-free broth with a lipolytic enzyme activity of 10 LU/ml was recovered after removal of the cell mass. This was deionized and freeze-dried to obtain 17.8 g of powder sample with a lipolytic enzyme activity of 3,050 LU/g.

Example 3

Purification and characterization of lipolytic enzyme from *Botryosphaeria sp.*

Purification procedure

Crude lipolytic enzyme from *Botryosphaeria sp.* CBS 102.95 was purified using a 2-step protocol using anion exchange and hydrophobic chromatography, as follows.

Anion exchange chromatography

6.2 g of the lipolytic enzyme preparation from Example 1 was dissolved in 10 mM Tris-HCl, pH 7 and dialyzed against the same buffer. The solution was applied onto a column packed with Q Sepharose (product of Pharmacia) equilibrated with the same buffer at a flow rate of 5 ml/min. Unbound material was washed with the same buffer, and the lipolytic enzyme was eluted with a linear gradient of 0-1 M NaCl concentration in the same buffer. Obtained fractions were assayed for lipolytic enzyme activity and pooled. The yield of this step was 54%.

Hydrophobic chromatography

To the lipolytic enzyme pool, ammonium acetate was added to give a final concentration of 0.8 M. The solution was applied to a column packed with Butyl Sepharose (product of Pharmacia) previously equilibrated with 0.8 M ammonium acetate. After thoroughly washing with a large amount of the same buffer, elution was done firstly with milli-Q-water and secondly with 30% isopropanol. The water fraction contained 59% of the applied activity, and the isopropanol fraction contained 27%.

Summary of the purification

Step	Activity (LU)	Yield (%)
Powder	22,000	100
Q Sepharose	11,850	54
Butyl Sepharose		
water pool	6,990	32
isopropanol pool	3,200	15

Characterization

Purity and molecular weight of the lipolytic enzyme were analyzed using a 4.20% gradient SDS-PAGE gel (Novex) under standard conditions. Molecular weight marker proteins were purchased from Pharmacia. After electrophoresis, proteins were stained with Coomassie brilliant blue. The molecular weight of the lipolytic enzyme from both the water pool and the isopropanol were found to be 55,000.

The pI of the lipolytic enzyme was measured using Ampholine PAG plate, pH 3.5-9.5 (product of Pharmacia). pI marker proteins from Pharmacia were used. The pI of the lipolytic enzyme from both pools was pI 3.8.

These results indicated that the lipolytic enzyme from the two pools obtained from Butyl Sepharose is the same. The lipolytic enzyme from the water pool was chosen for use in the characterization due to the higher purity.

The specific activity of the lipolytic enzyme was found to be about 400 LU/A_{280} (protein content determined by absorbance at 280 nm).

Example 4

Purification and characterization of lipolytic enzyme from *Botryosphaeria ribis*

Purification procedure

Crude lipolytic enzyme from *Botryosphaeria ribis* CBS 504.94 was purified by 3 steps as follows.

STREAM LINE™ column chromatography

The first step was STREAM LINE column chromatography. 14.3 g of the freeze-dried powder from Example 2 and 3.5 g of a another freeze-dried powder (made in essentially the same manner as in Example 2 and having a lipolytic enzyme activity of 1780 LU/g) were dissolved in 50 mM Tris-HCl buffer (pH 7.6). The lipolytic enzyme was adsorbed on a column of DEAE resin equilibrated with the same buffer, and the column was washed with the same buffer. The lipolytic enzyme was eluted with the same buffer including 0.5 M NaCl. The yield of this step was 45%.

Hydrophobic column chromatography

The second step was hydrophobic column chromatography using pre-packed Butyl Toyopearl (product of Toyo Soda) and HPLC. The concentrated lipolytic enzyme was adjusted to a salt concentration of 1 M ammonium acetate. Elution was carried out by a linear gradient of 1-0 M ammonium acetate and 20% ethanol. The fractions showing lipolytic enzyme activity were gathered. Ultrafiltration was performed to concentrate and desalt. The yield of this step was 89%.

Anion exchange column chromatography

The third step was anion exchange column chromatography using pre-packed DEAE Toyopearl (product of Toyo Soda). The lipolytic enzyme was adjusted to pH 7.6 and 0.1 M NaCl. This was applied to the column equilibrated with 50 mM Tris-HCl buffer (pH 7.6) including 0.1 M NaCl, and the lipolytic enzyme was eluted with a linear gradient of 0.1-2 M NaCl. The chromatogram showed two peaks with lipolytic enzyme activity. The fractions corresponding to the first peak were gathered and dialyzed for use in characterization of the lipolytic enzyme.

Summary of purification

Step	Activity (LU)	Yield (%)	
Powder	64615		100
STREAM LINE	29070	44.9	44.9
Butyl Toyopearl	26814	92.2	41.4
DEAE Toyopearl	4984	18.6	7.7

Molecular weight

The molecular weight of the purified lipolytic enzyme was calculated by SDS-PAGE and gel filtration column chromatography. SDS-PAGE was carried out using a 10-15 gradient gel (product of Pharmacia) and Phast System™ under standard conditions. Molecular weight marker proteins were purchased from Pharmacia. After hydrolysis, proteins were stained with Coomassie brilliant blue. The molecular weight of the lipolytic enzyme was found to be 64,000.

Gel filtration chromatography was carried out with Superdex 200pg 26/60 (product of Pharmacia) and HPLC. 2 ml of purified lipolytic enzyme was applied onto the column equilibrated with 50 mM Tris-HCl buffer including 0.1 M NaCl, and lipolytic enzyme was eluted with the same buffer. The flow rate was 3 ml/min. Gel Filtration Calibration Kit (product of Pharmacia) was used as the standard proteins. The molecular weight was found to be 54,000.

Iso-electric point

The pI of the lipolytic enzyme was determined by IEF-PAGE using a 3-9 gradient gel and Phast System™. pI marker proteins from Pharmacia were used. After electrophoresis, there was one band below pH 3.5 with the Coomassie brilliant blue staining. The lipolytic enzyme activity was found at the same position using olive oil emulsion and brilliant green.

Specific activity

The specific activity was found to be about 300 LU/mg. The protein amount was measured by protein assay kit, and Bovine Plasma Globulin Lyophilized was used as standard (product of Bio-Rad).

Example 5

Production of lipolytic enzyme from various strains

Each of the strains listed below was cultivated on an agar slant. About 1 cm^2 of the slant culture was scraped off and used to inoculate 100 ml of YS-2 medium in 500 ml shake flasks with two baffles. This seed culture was incubated at 30°C with shaking (approx. 220 rpm) for 2 days.

A main culture was prepared by inoculating 3 ml of the seed culture into 100 ml of YS-25 in 500 ml shake flasks with two baffles and cultivating at 30°C with shaking (approx. 220 rpm). The cultivation was continued for 3 days, except that it was extended to 6 days for the strains NN102563 and NN102564 because they were observed to grow more slowly than the other strains. At the end of cultivation, the lipolytic enzyme activity of the broth was measured.

Each experiment was carried out twice, and the results (average of the two experiments) were as follows:

Species	Strain No.	Lipolytic enzyme activity (LU/ml)
B. dothidea	NN102558	1.5
B. dothidea	NN102559	1.6
B. dothidea	NN102560	1.4
B. dothidea	NN102561	1.4
B. dothidea	NN102562	14.5
G. laricina	NN102563	0.2

G. laricina	NN102564	1.4
B. berengeriana	NN102565	1.1
B. berengeriana f. sp. pilicola	NN102566	1.3
G. paulowniae	NN102567	5.3
Botryosphaeria sp.	NN143554	27.0
B. ribis	NN115210	4.8
B. parva	NN103321	4.8
B. ribis	NN103322	1.7
B. ribis var. chromogena	NN103313	4.2
B. xanthocephala	NN103324	3.3

It is seen that lipolytic enzyme could be obtained from all the strains tested. It is further seen that the yield with the strain *Botryosphaeria* NN143554 (CBS 102.95) which was isolated by the inventors is remarkably higher than with other strains.

Example 6

Plate test for lipolytic enzyme activity at pH 10

The plate test described in Example 11 of WO 88/02775 (corresponding to JP-W 1-501120) was used to check for lipolytic enzyme activity at pH 10 with and without the addition of Ca^{++}, using culture broth from the previous example. The samples from all strains tested in Example 5 were found to exhibit lipolytic enzyme activity at pH 10, both with and without Ca^{++} addition.

Example 7

Determination of partial amino acid sequences

Purified lipases from *B. ribis* NN115210 and *Botryosphaeria sp.* NN143554 were subjected to sequencing.

No N-terminal amino acid sequences were obtained by direct sequencing of the two lipases. This shows that the N-terminal amino-group is blocked.

The two lipases were reduced and S-carboxymethylated before degradation with a lysyl-specific protease. The resulting peptides were fractionated and repurified using reversed phase HPLC before subjected to N-terminal amino acid sequencing.

5 peptide sequences were obtained from the NN115210 lipase. They are shown in the sequence listings as SEQ ID NO: 1 to 5.

The Xaa residue in SEQ ID NO: 4 is probably a glycosylated Asn residue.

One peptide sequence was obtained from the NN143554 lipase. This amino acid sequence was identical to the amino acid sequence shown in SEQ ID NO: 1 from the NN115210 lipase.

The peptide sequences were aligned to the known sequence of the lipase LIP 1 from *Candida cylindracea* CBS 6330 which has 534 amino acids. The five peptides were found to align to the following positions of LIP 1. The number of matching amino acids and the total amino acids in the peptide are given in parentheses. A gap was inserted between positions 373-374 of the LIP 1 sequence to improve the alignment.

SEQ ID NO: 1: positions 35-47 (12/13)

SEQ ID NO: 2: positions 148-187 (22/40)

SEQ ID NO: 3: positions 255-276 (7/22)

SEQ ID NO: 4: positions 296-331 (11/36)

SEQ ID NO: 5: positions 365-403 (18/40, gap inserted)

Based on this alignment, it is believed that the 5 peptides are partial sequences occurring in this order.

SEQUENCE LISTING

(1) GENERAL INFORMATION:

(i) APPLICANT:
(A) NAME: Novo Nordisk A/S
(B) STREET: Novo Alle
(C) CITY: Bagsvaerd
(E) COUNTRY: Denmark
(F) POSTAL CODE (ZIP): DK-2880
(G) TELEPHONE: +45-4444-8888
(H) TELEFAX: +45-4449-3256

(ii) TITLE OF INVENTION: Alkaline Lipolytic Enzyme

(iii) NUMBER OF SEQUENCES: 5

(iv) COMPUTER READABLE FORM:
(A) MEDIUM TYPE: Floppy disk
(B) COMPUTER: IBM PC compatible
(C) OPERATING SYSTEM: PC-DOS/MS-DOS
(D) SOFTWARE: PatentIn Release #1.0, Version #1.30 (EPO)

(2) INFORMATION FOR SEQ ID NO: 1:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 13 amino acids
(B) TYPE: amino acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: peptide

(v) FRAGMENT TYPE: internal

(vi) ORIGINAL SOURCE:
(A) ORGANISM: Botryosphaeria ribis
(B) STRAIN: CBS 504.94

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 1:

Gly Ile Pro Phe Ala Gln Pro Pro Val Gly Pro Leu Arg
1 5 10

(2) INFORMATION FOR SEQ ID NO: 2:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 40 amino acids
(B) TYPE: amino acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: peptide

(v) FRAGMENT TYPE: internal

(vi) ORIGINAL SOURCE:
(A) ORGANISM: Botryosphaeria ribis
(B) STRAIN: CBS 504.94

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 2:

Asp Ile Ile Phe Val Ala Val Asn Tyr Arg Val Gly Gly Phe Gly Phe
1 5 10 15

Leu Pro Gly Glu Glu Leu Gln Arg Asp Gly Ser Thr Asn Leu Gly Leu
20 25 30

Arg Asp Gln Arg Leu Ala Leu Glu
35 40

(2) INFORMATION FOR SEQ ID NO: 3:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 22 amino acids
(B) TYPE: amino acid
(C) STRANDEDNESS: single

(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: peptide

(v) FRAGMENT TYPE: internal

(vi) ORIGINAL SOURCE:
(A) ORGANISM: Botryosphaeria ribis
(B) STRAIN: CBS 504.94

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 3:

Ala Gln Asn Ile Tyr Asn Thr Val Val Glu Ser Ala Gly Cys Ser Gly
1 5 10 15

Ser Ser Asp Thr Leu Asn
20

(2) INFORMATION FOR SEQ ID NO: 4:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 36 amino acids
(B) TYPE: amino acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: peptide

(v) FRAGMENT TYPE: internal

(vi) ORIGINAL SOURCE:
(A) ORGANISM: Botryosphaeria ribis
(B) STRAIN: CBS 504.94

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 4:

Ile Phe Gly Tyr Arg Ser Leu Asp Leu Ser Tyr Leu Pro Arg Pro Asp
1 5 10 15

Pro Ser Asp Asn Phe Tyr Ser Glu Ser Pro Asp Val Xaa Val Thr Ala
20 25 30

Gly Arg Phe Ala
35

(2) INFORMATION FOR SEQ ID NO: 5:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 40 amino acids
(B) TYPE: amino acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: peptide

(v) FRAGMENT TYPE: internal

(vi) ORIGINAL SOURCE:
(A) ORGANISM: Botryosphaeria ribis
(B) STRAIN: CBS 504.94

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 5:

Ser Tyr Phe Pro Ala Thr Asp Pro Gln Val Val Ala Asp Leu Val Ala
1 5 10 15

Ser Tyr Pro Asn Asn Ile Pro Ala Gly Ser Pro Phe Arg Thr Gly Val
20 25 30

Leu Asn Glu Ile Arg Pro Gln Phe
35 40

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INDICATIONS RELATING TO A DEPOSITED MICROORGANISM

(PCT Rule 13*bis*)

A. The indications made below relate to the microorganism referred to in the description on page 3, lines 14 - 24.

B. IDENTIFICATION OF DEPOSIT — Further deposits are identified on an additional sheet [X]

Name of depositary institution

Centraal Bureau voor Schimmelcultures

Address of depositary institution *(including postal code and country)*

Oosterstraat 1, 3740 AG Baarn, Netherlands

Date of deposit	Accession Number
January 6, 1995	CBS 102.95

C. ADDITIONAL INDICATIONS *(leave blank if not applicable)* — This information is continued on an additional sheet [X]

In respect of those designations in which a European and/or Australian patent is sought, during the pendency of the patent application a sample of the deposited microorganism is only to be provided to an independent expert nominated by the person requesting the sample (Rule 28(4) EPC/Regulation 3.25 of Australia Statutory Rules 1991 No 71).

D. DESIGNATED STATES FOR WHICH INDICATIONS ARE MADE *(if the indications are not for all designated States)*

E. SEPARATE FURNISHING OF INDICATIONS *(leave blank if not applicable)*

The indications listed below will be submitted to the International Bureau later *(specify the general nature of the indications e.g., "Accession Number of Deposit")*

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32

INDICATIONS RELATING TO A DEPOSITED MICROORGANISM

(PCT Rule 13*bis*)

A. The indications made below relate to the microorganism referred to in the description on page 3, line s 14 - 24.

B. IDENTIFICATION OF DEPOSIT — Further deposits are identified on an additional sheet ☐

Name of depositary institution

Centraal Bureau voor Schimmelcultures

Address of depositary institution *(including postal code and country)*

Oosterstraat 1, 3740 AG Baarn, Netherlands

Date of deposit	Accession Number
October 19, 1994	CBS 504.94

C. ADDITIONAL INDICATIONS *(leave blank if not applicable)* — This information is continued on an additional sheet ☒

In respect of those designations in which a European and/or Australian patent is sought, during the pendency of the patent application a sample of the deposited microorganism is only to be provided to an independent expert nominated by the person requesting the sample (Rule 28(4) EPC/Regulation 3.25 of Australia Statutory Rules 1991 No 71).

D. DESIGNATED STATES FOR WHICH INDICATIONS ARE MADE *(if the indications are not for all designated States)*

E. SEPARATE FURNISHING OF INDICATIONS *(leave blank if not applicable)*

The indications listed below will be submitted to the International Bureau later *(specify the general nature of the indications e.g., "Accession Number of Deposit")*

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CLAIMS

1. An alkaline lipolytic enzyme which

a) is immunologically reactive with an antibody raised against a purified lipolytic enzyme produced by a strain of *Botryosphaeria* or *Guignardia*, and

b) has optimum activity at a pH in the range 9-11 in the presence of 50 mM Ca^{++}.

2. The lipolytic enzyme of claim 1 wherein the strain belongs to *Botryosphaeria*, preferably *B. berengeriana*, *B. berengeriana f. sp. pilicola*, *B. dothidea*, *B. parva B. ribis*, *B. ribis var. chromogena* or *B. xanthocephala*.

3. The lipolytic enzyme of claim 2 wherein the strain is *B. berengeriana* MAFF 06-45001, *B. berengeriana f. sp. pilicola* MAFF 06-45002, *B. dothidea* JCM 2733, JCM 2735, JCM 2736, JCM 2737, JCM 2738, *B. parva* ATCC 58191, *B. ribis* CBS 504.94, ATCC 56125, *B. ribis var. chromogena* CBS 121.26, *B. xanthocephala* ATCC 60638 or *Botryosphaeria sp.* CBS 102.95.

4. The lipolytic enzyme of claim 1 wherein the strain belongs to *Guignardia*, preferably *G. laricina* or *G. paulowniae*.

5. The lipolytic enzyme of claim 4 wherein the strain is *G. laricina* IFO 7887, IFO 7888 or *G. paulowniae* MAFF 03-05151.

6. An alkaline lipolytic enzyme which contains an amino acid sequence selected from the group consisting of SEQ ID NO: 1, 2, 3, 4 and 5.

7. The lipolytic enzyme of claim 6 wherein the sequence contains all of said sequences, preferably in the stated order.

8. The lipolytic enzyme of any of claims 1-7 provided as a detergent additive in the form of a non-dusting granulate, a stabilized liquid, a slurry, or a protected enzyme.

9. A biologically pure culture of *Botryosphaeria sp.* CBS 102.95 or a lipolytic enzyme-producing mutant thereof.

10. A method for producing a lipolytic enzyme, comprising cultivation of a lipolytic enzyme-producing strain of *Botryosphaeria* or *Guignardia* in a suitable nutrient medium, followed by recovery of the alkaline lipolytic enzyme.

11. The method of claim 10, wherein the lipolytic enzyme is an alkaline lipolytic enzyme having optimum activity at a pH in the range 9-11 in the presence of 50 mM Ca^{++}.

12. The method of claim 10 or 11, wherein the strain is *B. berengeriana* MAFF 06-45001, *B. berengeriana f. sp. pilicola* MAFF 06-45002, *B. dothidea* JCM 2733, JCM 2735, JCM 2736, JCM 2737, JCM 2738, *B. parva* ATCC 58191, *B. ribis* CBS 504.94, ATCC 56125, *B. ribis var. chromogena* CBS 121.26, *B. xanthocephala* ATCC 60638, *Botryosphaeria sp.* CBS 102.95, *G. laricina* IFO 7887, IFO 7888 or *G. paulowniae* MAFF 03-05151.

13. A method for producing a lipolytic enzyme, comprising:

a) isolating a DNA sequence encoding the lipolytic enzyme from a lipolytic enzyme-producing strain of *Botryosphaeria* or *Guignardia*,

b) combining the DNA fragment with appropriate expression signal(s) in an appropriate vector,

c) transforming a suitable heterologous host organism with the vector,

d) cultivating the transformed host organism under conditions leading to expression of the lipolytic enzyme, and

e) recovering the lipolytic enzyme from the culture medium.

14. The method of claim 13, wherein the lipolytic enzyme is an alkaline lipolytic enzyme having optimum activity at a pH in the range 9-11 in the presence of 50 mM Ca^{++}.

15. The method of claim 13 or 14, wherein the lipolytic enzyme-producing strain is *B. berengeriana* MAFF 06-45001, *B. berengeriana f. sp. pilicola* MAFF 06-45002, *B. dothidea* JCM 2733, JCM 2735, JCM 2736, JCM 2737, JCM 2738, *B. parva* ATCC 58191, *B. ribis* CBS 504.94, ATCC 56125, *B. ribis var. chromogena* CBS 121.26, *B. xanthocephala* ATCC 60638, *Botryosphaeria sp.* CBS 102.95, *G. laricina* IFO 7887, IFO 7888 or *G. paulowniae* MAFF 03-05151.

16. The method of any of claims 13-15, wherein the host organism is a filamentous fungus, preferably a strain of the genus *Aspergillus*.

17. The method of any of claims 13-15, wherein the DNA sequence is isolated by a method comprising:

a) cloning, in suitable vectors, a cDNA library from the lipolytic enzyme-producing strain of *Botryosphaeria* or *Guignardia*,

b) transforming suitable yeast host cells with said vectors,

c) cultivating the transformed yeast host cells under suitable conditions to express the alkaline lipolytic enzyme,

d) screening for positive clones by determining the lipolytic enzyme activity expressed in step (c).

18. A detergent composition comprising a surfactant and an alkaline lipolytic enzyme which

a) is immunologically reactive with an antibody raised against a purified lipolytic enzyme produced by a strain of *Botryosphaeria* or *Guignardia*, and

b) has optimum activity at a pH in the range 9-11 in the presence of 50 mM Ca^{++}.

19. A detergent composition comprising a surfactant and an alkaline lipolytic enzyme which contains an amino acid sequence selected from the group consisting of SEQ ID NO: 1, 2, 3, 4 and 5.

INTERNATIONAL SEARCH REPORT

International application No.
PCT/DK 96/00123

A. CLASSIFICATION OF SUBJECT MATTER

IPC6: C12N 9/20 // C11D 3/386
According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)

IPC6: C12N

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

SE,DK,FI,NO classes as above

Electronic data base consulted during the international search (name of data base and, where practicable, search terms used)

WPI, CA, BIOSIS, MEDLINE, EMBL, GENESEQ, PIR, SWISSPROT

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	EP 0258068 A2 (NOVO INDUSTRI A/S), 2 March 1988 (02.03.88), figures 1-4	1-19
X	EP 0385401 A1 (OCCIDENTAL CHEMICAL CORPORATION), 5 Sept 1990 (05.09.90), abstract	1-19
X	WO 9414940 A1 (NOVO NORDISK A/S), 7 July 1994 (07.07.94), abstract	1-19
X	EP 0218272 A1 (GIST-BROCADES N.V.), 15 April 1987 (15.04.87), abstract	1-19

[X] Further documents are listed in the continuation of Box C. [X] See patent family annex.

* Special categories of cited documents:
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"O" document referring to an oral disclosure, use, exhibition or other means
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Date of the actual completion of the international search	Date of mailing of the international search report
4 July 1996	17 -07- 1996
Name and mailing address of the ISA/ Swedish Patent Office Box 5055, S-102 42 STOCKHOLM Facsimile No. +46 8 666 02 86	Authorized officer Yvonne Siösteen Telephone No. +46 8 782 25 00

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INTERNATIONAL SEARCH REPORT

International application No.
PCT/DK 96/00123

C (Continuation). DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	Nucleic Acid Research, Pir 2 Accession no. Jn0552, Lotti,M. et al: "Cloning and analysis of Candida cylindracea lipase sequences", Gene 124, 45-55, 1993	6,19
X	Nucleic Acud Research, Pir 2 Accession no. S47655, Sone, T. et al: "Cloning and sequence analysis of a hamster liver cDNA encoding a novel putative carboxylesterase", Biochim. Biophys. Acta 1207, 138-142, 1994	6,19
A	Chemical Abstracts, Volume 95, No 21, 23 November 1981 (23.11.81), (Columbus, Ohio, USA), Shi, Qiao Qin, "Studies on alkaline lipase. 1. Screening and purification of the microorganisms", page 322, THE ABSTRACT No 183581u, Wei Sheng Wu Hsueh Tung Pao 1981, 8 (3), 108-110	1-19
A	EP 0271152 A2 (UNILEVER NV), 15 June 1988 (15.06.88)	1-19
A	Chemical Abstracts, Volume 92, No 5, 4 February 1980 (04.02.80), (Columbus, Ohio, USA), Aisaka, Kazuo et al, "Studies on lipoprotein lipases from microorganisms. Part I. Production of lipoprotein lipase and lipase by Rhizopus japonicus", page 391, THE ABSTRACT No 37376c, Agric. Biol. Chem. 1979, 43 (10), 2125-2129	1-19
A	EP 0238023 A2 (NOVO INDUSTRI A/S), 23 Sept 1987 (23.09.87)	13-17
A	Nucleic Acid Research, Pir 2 Accession no. Jn0553, Lotti, M. et al: "Cloning and analysis of Candida cylindracea lipase sequences", Gene 124, 45-55, 1993	6,19

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INTERNATIONAL SEARCH REPORT

International application No.
PCT/DK 96/00123

C (Continuation). DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
A	Chemical Abstracts, Volume 116, No 1, 6 January 1992 (06.01.92), (Columbus, Ohio, USA), Park, Seok Hee et al, "Production of pectolytic enzymes by Botryosphaeria dothidea", page 327, THE ABSTRACT No 3309g, Han'guk Kyunhakhoechi 1991, 19 (2), 143-147	9

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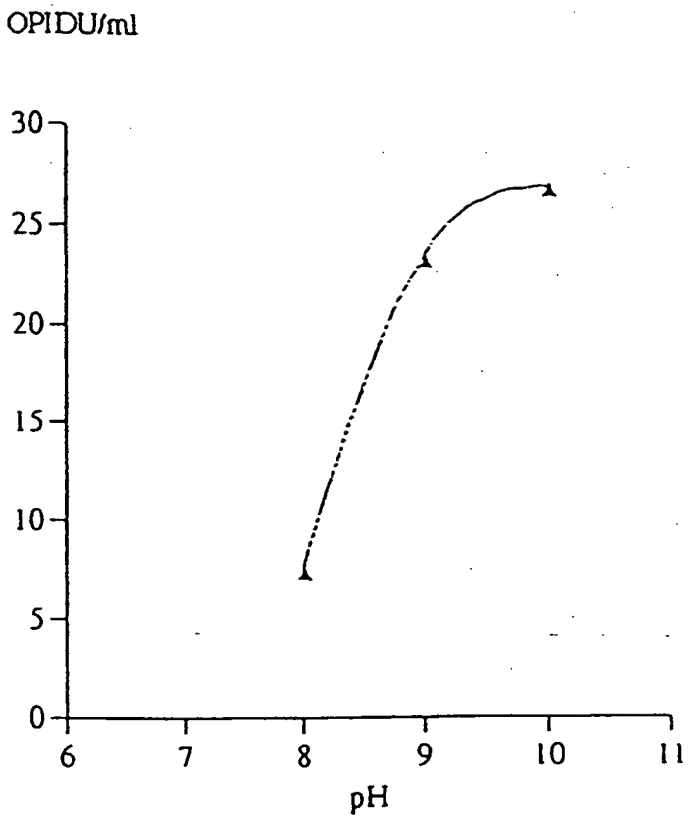

FIG. 1

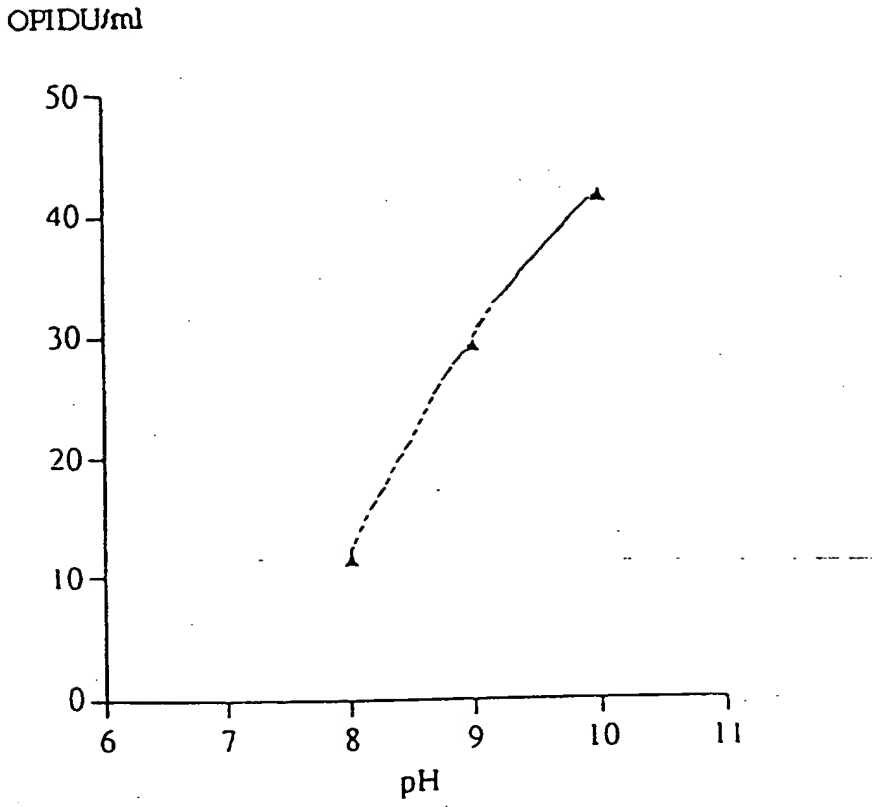

FIG. 2

INTERNATIONAL SEARCH REPORT

Information on patent family members

01/04/96

International application No.
PCT/DK 96/00123

Patent document cited in search report	Publication date	Patent family member(s)	Publication date
EP-A2- 0258068	02/03/88	SE-T3- 0258068	
		AT-T- 110768	15/09/94
		DE-D,T- 3750450	05/01/95
		ES-T- 2058119	01/11/94
		JP-B- 6033417	02/05/94
		JP-A- 63068697	28/03/88
		US-A- 4810414	07/03/89
EP-A1- 0385401	05/09/90	CA-A- 2010986	27/08/90
		JP-A- 3043073	25/02/91
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		CA-A- 1313360	02/02/93
		DE-A- 3684398	23/04/92
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		JP-T- 63500423	18/02/88
		NO-B,C- 176108	24/10/94
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		US-A- 5278066	11/01/94
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		DE-D,T- 3788524	11/05/94
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		ES-T- 2061446	16/12/94
		IE-B- 63169	22/03/95
		JP-A- 7051067	28/02/95
		JP-A- 62272988	27/11/87

Box No. VI PRIORITY CLAIM Further priority claims are indicated in the Supplemental Box ☐

The priority of the following earlier application(s) is hereby claimed:

Country (in which, or for which, the application was filed)	Filing Date (day/month/year)	Application No.	Office of filing (only for regional or international application)
item (1) DK	30 March 1995 (30/03/95)	344/95	
item (2) DK	14 July 1995 (14/07/95)	830/95	
item (3)			

Reference 4406.204 Country GB

SLK 9.9.96

Mark the following check-box if the certified copy of the earlier application is to be issued by the Office which for the purposes of the present international application is the receiving Office (a fee may be required):

☒ The receiving Office is hereby requested to prepare and transmit to the International Bureau a certified copy of the earlier application(s) identified above as item(s): (1), (2)

Box No. VII INTERNATIONAL SEARCHING AUTHORITY

Choice of International Searching Authority (ISA) *(If two or more International Searching Authorities are competent to carry out the international search, indicate the Authority chosen; the two-letter code may be used):* ISA / SE

Earlier search *Fill in where a search (international, international-type or other) by the International Searching Authority has already been carried out or requested and the Authority is now requested to base the international search, to the extent possible, on the results of that earlier search. Identify such search or request either by reference to the relevant application (or the translation thereof) or by reference to the search request.*

Country (or regional Office): Date *(day/month/year)*: Number:

Box No. VIII CHECK LIST

This international application contains the following number of sheets:

1. request	:	5 sheets
2. description	:	32 sheets
3. claims	:	4 sheets
4. abstract	:	1 sheets
5. drawings	:	2 sheets
Total	:	44 sheets

This international application is accompanied by the item(s) marked below:

1. ☐ separate signed power of attorney
2. ☐ copy of general power of attorney
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6. ☐ separate indications concerning deposited microorganisms
7. ☒ nucleotide and/or amino acid sequence listing (diskette)
8. ☐ other *(specify)*:

Figure No. _____ of the drawings (if any) should accompany the abstract when it is published.

Box No. IX SIGNATURE OF APPLICANT OR AGENT

Next to each signature, indicate the name of the person signing and the capacity in which the person signs (if such capacity is not obvious from reading the request).

Sten L. Knudsen

Sten L. Knudsen, Patent Counsel
Novo Nordisk A/S

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1. Date of actual receipt of the purported international application: RO/DK 27 MAR 1996 (27.03.96)
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4. Date of timely receipt of the required corrections under PCT Article 11(2):
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Vor ref.: bib 1 jk 03278
Deres ref.: PCT/DK96/00123 Sten.L,Knudsen
Kundenr.: 000056

Dato: 07 maj 1996 Side: 1
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Faktura nr.: 185557

Antal	Tekst	Pris pr. stk.	Nettopris	Moms	Pris ialt
2	Bekræftet kopi, patenter	300,00	600,00		600,00
53	Fotokopi pr. side	3,25	172,25	43,06	215,31
1	Ekspeditionsgebyr	30,00	30,00	7,50	37,50

Prioritetsdokument 344/95 + 830/95
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